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Structural basis for the substrate selectivity of a HAD phosphatase from *Thermococcus onnurineus* NA1

Tri Duc Ngo [a], Binh Van Le [a], Vinod Kumar Subramani [a], Chi My Thi Nguyen [a], Hyun Sook Lee [b], Yona Cho [b], Kyeong Kyu Kim [a, *], Hye-Yeon Hwang [a, *]

[a] Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon 440-746, Republic of Korea
[b] Korea Institute of Ocean Science and Technology, Ansan 426-744, Republic of Korea

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ABSTRACT

Proteins in the haloalkaloic acid dehalogenase (HAD) superfamily, which is one of the largest enzyme families, is generally composed of a catalytic core domain and a cap domain. Although proteins in this family show broad substrate specificities, the mechanisms of their substrate recognition are not well understood. In this study, we identified a new substrate binding motif of HAD proteins from structural and functional analyses, and propose that this motif might be crucial for interacting with hydrophobic rings of substrates. The crystal structure of TON_0338, one of the 17 putative HAD proteins identified in a hyperthermophilic archaeon, *Thermococcus onnurineus* NA1, was determined as an apo-form at 2.0 Å resolution. In addition, we determined the crystal structure TON_0338 in complex with Mg^{2+} or N-cyclohexyl-2-aminoethanesulfonic acid (CHES) at 1.7 Å resolution. Examination of the apo-form and CHES-bound structures revealed that CHES is sandwiched between Trp58 and Trp61, suggesting that this Trp sandwich might function as a substrate recognition motif. In the phosphatase assay, TON_0338 was shown to have high activity for flavin mononucleotide (FMN), and the docking analysis suggested that the flavin of FMN may interact with Trp58 and Trp61 in a way similar to that observed in the crystal structure. Moreover, the replacement of these tryptophan residues significantly reduced the phosphatase activity for FMN. Our results suggest that WxxW may function as a substrate binding motif in HAD proteins, and expand the diversity of their substrate recognition mode.

1. Introduction

The haloalkaloic acid dehalogenase (HAD) superfamily is regarded as one of the largest enzyme families [1,2]. Though this family was originally named after haloacid dehalogenases, the majority of members mediate phosphoryl transfer and phosphatases are the most dominant among them [1–3]. HAD phosphatases act on a variety of substrates, but, the biological functions are not known for most of them [4]. Structural and biochemical studies on a number of HAD proteins have provided the atomic basis of their substrate specificity [3]. However, more intensive analyses are necessary to uncover their diversity in substrate-recognition modes and physiological roles.

Structurally characterized HAD phosphatases share a unique HAD fold in the core domain that is characterized by a three-layered α/β sandwich consisting of repeating α/β units [3]. The active site in the core domain is constructed of four loops with consensus motifs (motif I-IV). Motifs I, II, and IV contain key residues involved in nucleophilic attack, phosphoryl group binding, and Mg^{2+} binding, respectively [2,3]. Arg/Lys in Motif III stabilizes the negative charge of the Asp nucleophile and phosphoryl group.

In addition to the core domain, HAD proteins contain a cap domain that is known to mediate substrate binding. HAD proteins are classified into subfamilies C0, C1 and C2 based on the organization and location of cap domains [3]. While C0 structure has very small cap domains, C1 and C2 caps have α-helical bundle and mixed α/β structures of considerable sizes, respectively. The open and

Abbreviations: HAD, haloalkaloic acid dehalogenase; FMN, flavin mononucleotide; CHES, N-cyclohexyl-2-aminoethanesulfonic acid; β-PGM, β-phosphoglucomutase.

* Corresponding authors.
E-mail addresses: kyeongkyu@skku.edu (K.K. Kim), hyhwang3@gmail.com (H.-Y. Hwang).

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closed conformations, defined by the relative orientation between the core and cap domains, are required for substrate entry and hydrolysis [5].

The genome analyses of *Thermococcus onnurineus* NA1, a hyperthermophilic archaeon isolated from a deep-sea hydrothermal vent area, revealed 17 putative HAD proteins [6]. Their functions cannot be predicted from the sequence alone, thus structural and biochemical characterizations are necessary. Recent advances in structural genomics provide an opportunity to identify molecular functions of unknown proteins from structural information [7]; these approaches are expected to be applicable to HAD proteins. TON_0338, annotated as one of the HAD phosphatases in *T. onnurineus* NA1, contains four conserved motifs and a C1-type cap domain (Fig. 1). Structural studies of apo-form, Mg^{2+}- and CHES-bound TON_0338, combined with activity assay, revealed that TON_0338 has phosphatase activity with a preference for substrates containing hydrophobic ring moieties. Further docking and mutational analyses confirmed that the WxxW motif in the cap domain is crucial for substrate recognition, suggesting a novel substrate binding mode of HAD phosphatases.

2. Materials and methods

2.1. Protein expression and purification

The cloning and purification methods of the recombinant TON_0338 with a fused C-terminal His tag was described previously [8]. Briefly, The transformed *Escherichia coli* Rosetta(DE3)pLysS was harvested and sonicated in 50 mM Tris–HCl (pH 8.0), 0.5 M KCl, and 10% glycerol. Proteins were purified from the clarified cell lysates using a Hi-Trap chelating column and a Superdex 200 10/300 GL column (Amersham Biosciences) sequentially. The pooled fractions were concentrated using a Centricon YM-10 (Millipore) in 10 mM Tris–HCl (pH 8.0) and 50 mM NaCl. Selenomethionine-substituted protein, expressed in *E. coli* B834(DE3), was prepared in the same way.

2.2. Crystallization and data collection

The initial crystallization screening for native and selenomethionine (SeMet)-substituted TON_0338 proteins was performed by the microbatch crystallization method at 20 °C as described previously [8]. The diffraction-quality crystals of native and SeMet-substituted apo-TON_0338 were obtained using 35% t-butanol and 1.0 M trisodium citrate (pH 5.6). The Mg^{2+}-bound TON_0338 was crystallized using 20% PEG 3000 and 0.1 M citrate (pH 5.5) as precipitant. CHES-bound proteins were crystallized by mixing 1 μl of TON_0338, preincubated with 10 mM glucose 6-phosphate (G6P), with 1 μl of 1.0 M sodium citrate and 0.1 M CHES (pH 9.5). Crystals were transferred to a cryo-solution containing the original precipitant and 25% (v/v) ethylene glycol prior to flash-freezing in a cold nitrogen stream. All diffraction data were collected at beam line 4A of Pohang Accelerator Laboratory, South Korea. The diffraction data from the native, Mg^{2+}- and CHES-bound crystals were collected at 2.0, 1.7, and 1.7 Å resolutions, respectively, using synchrotron radiation at a wavelength of 1.0000 Å. The crystals of apo-TON_0338 belong to monoclinic space group C2 with unit cell $a = 121.2$ Å, $b = 63.0$ Å, $c = 37.5$ Å and $\beta = 106.5°$. The crystals of the

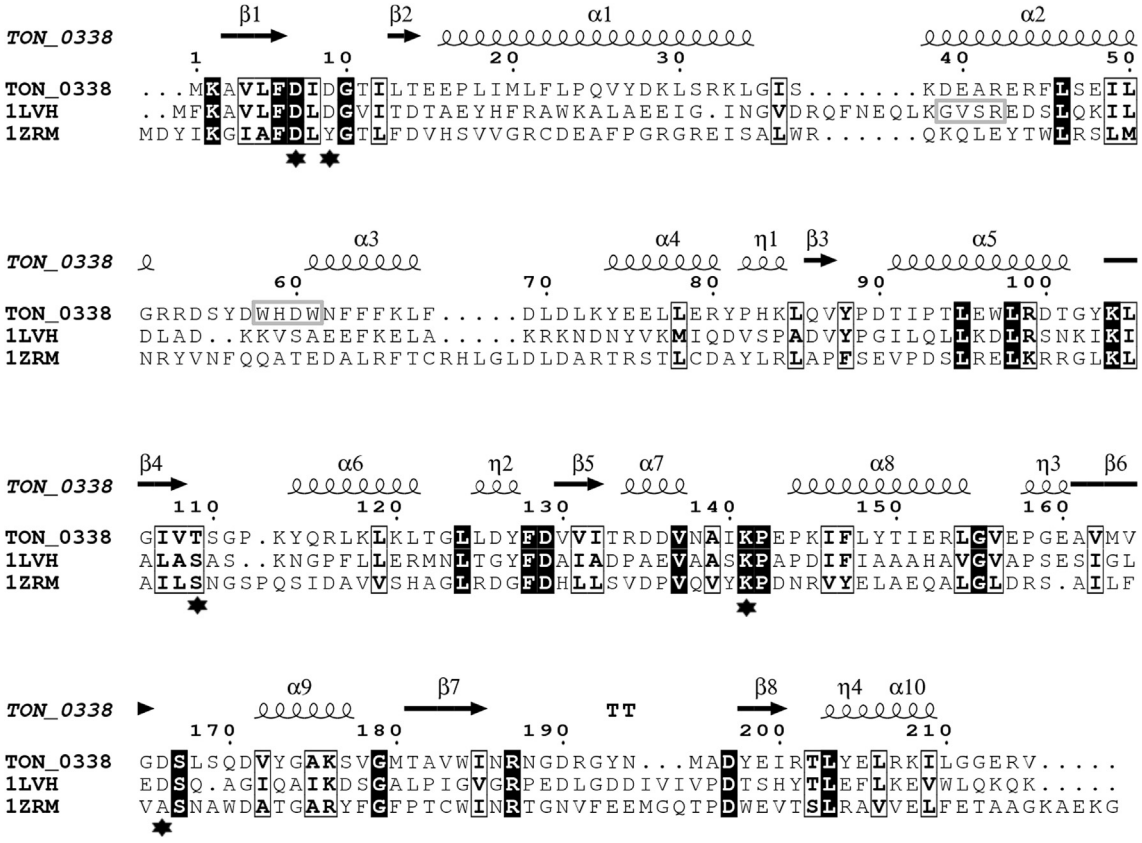

Fig. 1. Sequence alignment of TON_0338 from *Thermococcus onnurineus* NA1, *Lactococcus lactis β-phosphoglucomutase (1LVH)* and *Pseudomonas* sp. YL L-2-haloacid dehalogenase (1ZRM). Secondary structures of TON_0338 are shown above the line. Residues in four catalytic motifs are noted with asterisks. Well-conserved and similar residues are shown as bold white and bold black letters, respectively bold black letters. Stretches of residues that are similar across the group of sequences are shown in boxes. GxxR and WxxW motifs are also indicated with grey boxes.